Flow-mediated dilatation

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Arterial endothelial dysfunction is one of the key early events in atherogenesis, preceding structural atherosclerotic changes. It is also important in the late stages of obstructive atherosclerosis, predisposing to constriction and/or thrombosis. Endothelial function can be measured in coronary arteries and in the periphery by measuring vasomotor function after intra-arterial infusion of pharmacologic substances which enhance the release of endothelial nitric oxide. The disadvantage of these methods is their invasive nature, which generally makes them unsuitable for studies involving asymptomatic subjects. For this reason, noninvasive tests of endothelial function have been developed. In the most widely used of these, an ultrasound-based method, arterial diameter is measured in response to an increase in shear stress, which causes endothelium-dependent dilatation. Endothelial function assessed by this method correlates with invasive testing of coronary endothelial function, as well as with the severity and extent of coronary atherosclerosis. This noninvasive endothelial function testing has provided valuable insights into early atherogenesis, as well as into the potential reversibility of endothelial dysfunction by various strategies, including pharmacological agents (lipid lowering, ACE inhibition), L-arginine, antioxidants and hormones.

Keywords: endothelial function, flow-mediated dilatation, ultrasound

Introduction

Atherosclerosis is a diffuse process of the arterial tree that starts in childhood and early adult life [1]. Its 'preclinical' stage may last for decades, during which time atherosclerotic changes progress slowly, eventually causing lumenal stenosis and/or disruptive lesions leading to clinical symptoms. Endothelial dysfunction, especially reduction in the bioavailability of endothelium-derived nitric oxide, is a key early event in atherogenesis, appearing long before the formation of structural atherosclerotic changes [2, 3]. Nitric oxide is released from the endothelial cells in response to increased shear stress and certain pharmacologic stimuli [4]. Nitric oxide may function as an endogenous antiatherogenic molecule by maintaining low arterial tone at rest, inhibiting leucocyte–endothelial interactions, attenuating platelet aggregation and inhibiting smooth muscle cell proliferation [4]. Therefore the testing of endothelial nitric oxide release in humans allows investigation of one particularly important aspect of normal arterial physiology.

It is possible to measure endothelial function by measuring vasodilatation after intra-arterial pharmacologic stimulation with substances that enhance the release of endothelial nitric oxide (such as acetylcholine and bradykinin). The major disadvantage of these methods is their invasive nature, which makes them generally unsuitable for use in asymptomatic young subjects. Therefore, noninvasive tests of endothelial function have been developed. One based on ultrasound measures flow-mediated changes in arterial diameter in relatively superficial arteries, such as the brachial, radial or femoral vessels. Thus, this technique measures endothelial function in conduit arteries rather than resistance vessels. Flow-mediated changes in conduit artery diameter are caused

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by shear-stress induced generation of endothelial derived vasoactive mediators (flow-mediated dilatation, FMD). Since the arterial dilator response to shear-stress can be almost completely blocked by pretreatment with nitric oxide synthase inhibitors [5], it has been suggested that the phenomenon is predominantly due to endothelial release of nitric oxide. Correlation between FMD and agonist-induced endothelial function in microvessels has not yet been published. However, endothelial function assessed by this method correlates significantly with invasive testing of coronary endothelial function [5, 6], as well as with the severity and extent of coronary atherosclerosis [7]. Although not yet recommended for routine clinical use, noninvasive endothelial function testing has provided valuable insights into vascular changes associated with early atherogenesis and the potential reversibility of arterial disease.

Methodology

The diameter of the target artery is measured by high-resolution external vascular ultrasound in response to an increase in blood flow (causing shear-stress) during reactive hyperaemia (induced by cuff inflation and then deflation). This leads to endothelium-dependent dilatation; the response is contrasted with that to sublingual nitroglycerin, an endothelium-independent dilator. The artery is scanned and the diameter measured during three conditions; at baseline, during reactive hyperaemia (induced by inflation and then deflation of a sphygmomanometer cuff around the limb, distal to the scanned part of the artery) and finally after administration of sublingual nitroglycerin using a normal antianginal dose of 400 μg (which causes endothelium-independent smooth muscle mediated vasodilatation) (Figure 1). The time required to obtain a high quality baseline scan varies between 1 and 10 min. The cuff inflation period of 5 min was initially decided to produce adequate hyperaemia to allow flow-mediated dilatation, but not to compromise patient comfort. Shorter inflation periods do not seem to produce significant flow-mediated dilatation [8]. The usual scanning period used in our laboratory is 30 s before and 90 s after the cuff deflation.

The diameter of the target artery is measured from B-mode ultrasound images of the centre of the target artery, identified when the clearest picture of the anterior and posterior intimal layers is obtained. The focus is set to the depth of the near wall, in view of the difficulty of evaluating the near compared with the far wall 'm' line (the interface between media and adventitia). Depth and gain settings can be set to optimize images of the lumen–arterial wall interface, images are magnified using a resolution box function and machine operating parameters are kept constant during the studies. When a satisfactory transducer position is found, the skin is marked and the arm remains in the same position throughout the study [2].

Due to considerations of ultrasound frequency and wavelength, which determine the resolution of the method, transducers with a frequency greater than or equal to 7.0 MHz must be used. In principle, the shorter the wavelength the better the axial resolution. The ideal vessel size for this test is 2.5–5.0 mm. Arteries below 2.5 mm are difficult to image accurately and reproducibly, and very small changes in absolute diameter are reflected as large percentage changes. Vessels larger than 5 mm tend not to dilate significantly, even in the presence of normal endothelial function, due to the inverse relationship between endothelium-dependent dilatation and vessel size in normal arteries. Over 90% of normal individuals have brachial artery size less than 5 mm [9], so it is relatively rare for brachial vessels to be too large for scanning. Femoral arteries are too big to study in all but prepubertal children, in which group they may be the artery of choice. Radial arteries, on the other hand, are often smaller than the ideal vessel size.

This test appears to be accurate, with submillimetre distances being measurable reliably and reproducibly [10]. In the study by Sorensen *et al.* [10], the overall coefficient of variation in FMD was 1.8%. These data also showed the expected significant between patient variability, and also significant day to day variation, but little variation between weeks or months [10]. As normal premenopausal women show variations in FMD through the menstrual cycle [11], and FMD may change after high-fat meals [12], we recommend studying subjects when fasting, and controlling for menstrual cycle times in serial studies in younger women.

A number of variations of the original method have been described. Some investigators use stereotactic devices to fix the arm and/or the transducer, to enhance reliable and reproducible diameter measurements. Some laboratories use A-mode or radio-frequency signals to detect the edge of the vessel, rather than the two dimensional B-mode ultrasound images [5]. Cuff placement above or below the scanned part of the artery have been described, and varying duration and pressures for cuff inflation have been used [8]. The brachial artery has been the target artery in most studied, but radial and femoral arteries have also been measured. Due to these technical modifications, the normal ranges established in some laboratories differ from normal ranges observed in others. This makes it difficult to compare values for endothelium-dependent dilatation obtained in different laboratories. Most investigators have found this technique to be accurate and reproducible [10, 13, 14], although some groups have reported poor reproducibility [15].

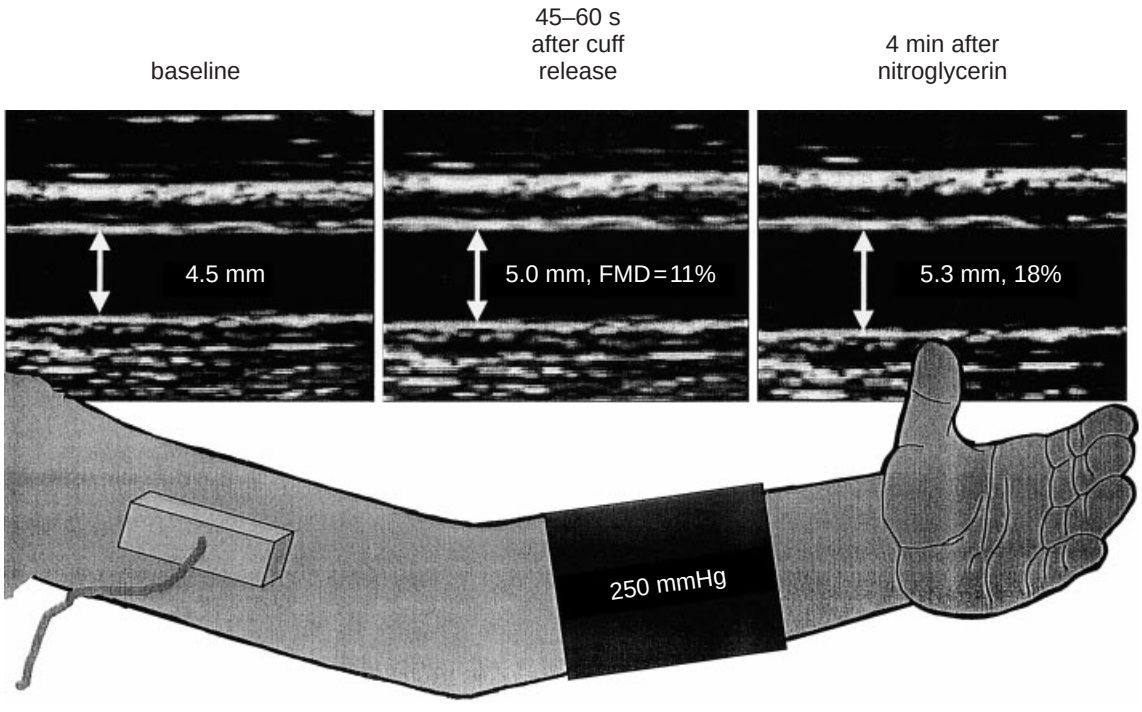

Figure 1 Brachial artery diameter is measured during three conditions; baseline (after at least 10 min supine rest), during reactive hyperaemia (induced by inflation to 250 mmHg and then deflation of a sphygmomanometer cuff around the forearm) and finally after the administration of sublingual nitroglycerin. A linear array, high resolution ultrasound transducer is used to provide B-mode images of the target vessel, proximal to the forearm cuff. FMD = flow-mediated dilatation.

Observational studies

In 1992, endothelial dysfunction was demonstrated in asymptomatic children and young adults with risk factors for atherosclerosis, such as hypercholesterolaemia and cigarette smoking [2]. In a subsequent study Sorensen *et al.* [16] found that familial hypercholesterolaemia is associated with arterial endothelial dysfunction in children as young as 6 years of age, with significant correlations between the degree of endothelial impairment and the levels of both LDL cholesterol and Lp(a). Furthermore, it has been shown that the traditional vascular risk factors identified from epidemiological studies (i.e. high cholesterol, smoking, older age) may interact to damage the endothelium in asymptomatic subjects, in the same way as they are known to interact in determining the risk of clinical cardiovascular endpoints [17]. Ageing too has been associated with progressive endothelial impairment and this age-related dysfunction occurs earlier in men than in women, consistent with a protective effect of oestrogens on the vessel wall [18]. Other risk factors associated with endothelial dysfunction using this technique include diabetes mellitus [19] and low HDL cholesterol [20].

Some observational studies have suggested that endothelial dysfunction might be reversible. For example, active cigarette smoking has been shown to be associated with impaired endothelium-dependent dilatation in a dose-dependent manner in otherwise healthy teenagers and young adults, and this impairment seems to partially reversible after smoking cessation [21]. Even exposure to environmental tobacco smoke (passive smoking) causes endothelial dysfunction [22], which is also potentially reversible, particularly after prolonged withdrawal from environmental tobacco smoke exposure [23].

Studies of reversibility

Once subjects with endothelial dysfunction have been identified, the next step involves studying potential strategies for disease reversibility. As the noninvasive ultrasound method for peripheral endothelial testing is generally accurate and reproducible, and can be performed serially, several investigators have used this method to address the question of reversibility of endothelial function.

Lipid lowering therapy

Many observational studies have documented a significant relationship between high serum cholesterol concentration and endothelial dysfunction, suggesting that lowering cholesterol concentration may result in improvement. Cholesterol lowering may improve endothelial function rapidly [24], and the vascular benefits appear to regress

quickly after treatment cessation [25]. The effect of cholesterol lowering on conduit artery endothelial function was studied by Vogel *et al.* [26], who treated healthy hypercholesterolaemic middle-aged men with simvastatin (10 mg daily). The mean FMD rose from a baseline value of $5.0 \pm 3.6\%$ to $15.7 \pm 4.9\%$ after 12 weeks therapy. Overall, the change in FMD correlated with the change in cholesterol levels ($r = 0.47$, $P = 0.004$). Simons *et al.* [27] investigated the effects of long-term (30 weeks) lipid lowering therapy on brachial FMD in 32 patients with primary hypercholesterolaemia. Both atorvastatin (80 mg daily) and simvastatin (40 mg daily) improved the median FMD from 2.2% to 5.5%, and from 1.8% to 4.5%, respectively. Contrary to these findings, Andrews *et al.* [28] did not observe any improvement in FMD in subjects with normal or mildly elevated LDL cholesterol levels and low HDL cholesterol levels treated with gemfibrozil (and niacin and/or cholestyramine to raise HDL cholesterol levels), despite significant improvements in lipid levels [28]. Thus, the highly significant improvement in endothelial function seen after statin therapy might not be entirely explained by the reduction in cholesterol levels. In keeping with this, O'Driscoll *et al.* [29] showed by using invasive plethysmography techniques that the beneficial effect on endothelial function induced by simvastatin was not correlated with the decrease in serum total cholesterol levels, and that the improvement in endothelial function seemed to continue even without further decrease in cholesterol concentration.

Angiotensin-converting enzyme (ACE) inhibitors

By using invasive methods, angiotensin-converting enzyme inhibitors have been shown to improve endothelial function in coronary arteries in patients with coronary artery disease [30], as well as in the forearm resistance vessels in patients with insulin-dependent diabetes mellitus [31]. Mullen *et al.* [32] studied the effect of angiotensin-converting enzyme inhibitor enalapril (20 mg once daily) on conduit endothelial function in 91 young subjects with insulin-dependent diabetes mellitus using a randomised, double-blind, parallel-group design. Brachial FMD was measured at baseline and after 12 and 24 weeks of treatment. In this study, treatment with enalapril had no significant effect on FMD. The authors suggested that the lack of improvement may reflect the complex nature of vascular disease in diabetes mellitus, probably affecting both endothelial and smooth muscle function. Studies are underway comparing the endothelial effects of different ACE inhibitor compounds. However, definitive results are not yet available.

Antioxidant vitamins and cellular redox state

Inactivation of endothelium derived nitric oxide due to increased production of oxygen free radicals in the vessel wall is thought to be an important mechanism for endothelial dysfunction [33]. As a result, much interest has focused on antioxidants, such as vitamin E and vitamin C, as they may scavenge free radicals and therefore improve endothelial function. A single oral dose of vitamin C (2 g) has been shown to improve brachial FMD acutely in patients with coronary artery disease [34], as has intravenous infusion of vitamin C in smokers [35]. Long-term effects of vitamin C on conduit artery endothelial function have not been studied extensively, but Hornig *et al.* [36] observed a significant improvement (from 8.2 ± 1.0 to $11.9 \pm 0.9\%$, $P < 0.05$) in radial FMD in five patients with chronic heart failure after 4 weeks high dose oral vitamin C supplementation (1 g twice daily). Furthermore, 4 week's combination therapy of oral vitamin C (1 g per day) and vitamin E (400 IU twice daily) has been shown to improve brachial FMD in children and adolescents with endothelial dysfunction due to hereditary hypercholesterolaemia [37]. In contrast, Simons *et al.* [38] recently documented a lack of benefit of long-term oral vitamin E therapy on arterial endothelial function in the healthy elderly.

It has been suggested that alterations in cellular redox state might be important in regulating endothelial derived nitric oxide action, and that these actions might explain the well documented acute effects of vitamin C on endothelial function. This hypothesis was tested by Vita *et al.* [39], who observed a significant effect of L-2-oxo-4-thiazolidine carboxylate on brachial FMD in 48 patients with coronary heart disease. This agent changes cellular redox state by increasing the amount of intracellular glutathione synthesis. Therefore, cellular redox state may be an important regulator of nitric oxide action and a determinant of arterial endothelial function.

Invasive studies in coronary vasculature have suggested that combining antioxidant vitamins with lipid-lowering therapy might have additive beneficial effects on endothelial function. Anderson *et al.* [40] found that the addition of probucol to lovastatin improved coronary endothelial function more than that with the statin alone. Neunteufl *et al.* [41] investigated whether lipid-lowering therapy in combination with vitamin E supplementation improves peripheral endothelial function more effectively than lipid-lowering therapy alone. In seven middle-aged patients with hypercholesterolaemia, the baseline FMD improved from $4.9 \pm 2.5\%$ to $16.4 \pm 4.7\%$ after 8 weeks of combination therapy (20 mg simvastatin and 300 IU vitamin E), and decreased significantly to $7.9 \pm 4.7\%$ after 4 weeks of withdrawal from vitamin E. Thus, the improvement of FMD seems to more pronounced after

a combination therapy with vitamin E than after lipid-lowering alone. Although some long-term studies have suggested beneficial effects of antioxidants on vascular function, there are still no clear data supporting the concept that long-term therapy with any antioxidant on its own is associated with improved endothelial function.

L-arginine

The amino acid L-arginine is the substrate for nitric oxide synthase, the enzyme which catalyses the production of nitric oxide in endothelial and other cells [42]. Experimental studies in cholesterol fed animals have shown that dietary supplementation with L-arginine improves endothelial function and reduces atherosclerosis [43]. Adams *et al.* [44] showed in a controlled cross-over trial that oral L-arginine (7 g 3 times daily) significantly improved brachial artery FMD in young men with coronary artery disease. After 3 day therapy with L-arginine, baseline FMD increased from 1.8 ± 0.7 to $4.7 \pm 1.1\%$ ($P < 0.04$). Clarkson *et al.* [45] studied the effects of 4 week oral L-arginine therapy (7 g 3 times daily) in asymptomatic hypercholesterolaemic young adults in controlled cross-over trial. FMD of the brachial artery improved from 1.7 ± 1.3 to $5.6 \pm 3.0\%$ ($P < 0.001$) during L-arginine supplementation, whereas placebo had no significant effect. Intravenous L-arginine acutely improved FMD in hypercholesterolaemic subjects (from 1.9 ± 1.9 to $4.1 \pm 2.1\%$, $P = 0.01$) and in smokers (from 2.0 ± 1.7 to $3.1 \pm 2.5\%$, $P = 0.02$), but not in diabetics [46]. In healthy young adults with preserved endothelial function, however, L-arginine does not seem to be effective in enhancing FMD [46, 47], suggesting that the production of nitric oxide of normally functioning endothelium cannot be increased by adding extra substrate. Thus, several controlled trials have now shown that endothelial dysfunction associated with hypercholesterolaemia or coronary artery disease can be successfully reversed by oral L-arginine therapy. Long-term data are awaited, however, some observations are encouraging [48].

Oestrogens

Oestrogen therapy in postmenopausal women is associated with lower the risk of cardiovascular events, and the augmented release on endothelium-derived nitric oxide by oestrogens has been suggested to be one of the mechanisms for the cardioprotective effects [49]. Most published studies have supported this hypothesis, showing beneficial effects of hormone replacement therapy on FMD in postmenopausal women. The beneficial effects of oestrogen therapy on FMD have also been documented in males taking high dose oestrogens (male to female transsexuals) [50, 51]. Lieberman *et al.* [52] studied 13 postmenopausal women in a placebo-controlled, cross-over trial. After 9 weeks of oral oestradiol therapy ($1\ mg\ day^{-1}$), baseline brachial FMD improved from 6.8% to 13.5%. Bush *et al.* [53] studied 18 postmenopausal women before and 1 and 6 months after receiving oral hormone replacement therapy (12 on oestrogen only, 6 on combination with progesterone). The baseline FMD increased from $0.4 \pm 7.1\%$ to $4.8 \pm 6.6\%$ after 1 month, and to $8.3 \pm 3.4\%$ after 6 months of therapy. McCrohon *et al.* [54] measured brachial FMD in postmenopausal women without therapy ($n = 40$) and in women who had been on hormone replacement therapy at least for 2 years ($n = 55$). FMD was significantly better in women taking hormones (6.2 ± 3.3 *vs* 4.4 ± 3.4, $P = 0.01$) and there were no differences in FMD in subgroups taking oestrogen alone ($n = 40$) or in combination with progesterone ($n = 15$).

It has been suggested that a combination therapy with progesterone might not effectively improve endothelial dysfunction, due to attenuation by progesterone. Gerhard *et al.* [55] studied 17 postmenopausal women in placebo-controlled cross-over trial using transdermal estradiol, with or without vaginal progesterone. During oestradiol therapy FMD was $11.1 \pm 1.1\%$ compared to 4.7 ± 0.6 on placebo ($P < 0.001$). Progesterone did not significantly attenuate this improvement. In a large study by Sorensen *et al.* [56], however, 100 healthy postmenopausal women were randomised to combined hormone replacement therapy or no substitution. In contrast to earlier observations, these investigators were not able to observe any significant improvement in FMD in the treatment group after a mean follow-up of 2.9 ± 0.5 years. The type and route of administration of the progestogen may be an important factor in these apparently conflicting results.

Folic acid

Homocysteine is damaging to endothelial cells in animal and cell culture studies, and high levels of homocysteine [57] have also been associated with impaired endothelial function in humans. Folic acid lowers serum homocysteine levels, and treatment with folic acid has been used succesfully to improve endothelial function in adults with hyperhomocysteinemia [58].

Diet and physical activity

The effects of life-style factors on endothelial function have received less attention. Among dietary factors are omega-3 fatty acids, which may improve endothelial function in patients with coronary artery disease. Kothny *et al.* [59] investigated the short-term effects of omega-3 fatty acids on radial FMD. They gave $18\ g\ day^{-1}$ fish oil concentrate (6.4 g eicosapentaenoic acid and 3.9 g

docosahexaenoic acid) in 18 patients with coronary artery disease in a randomised, placebo controlled study. No significant improvements, however, were observed in endothelial function either after active or placebo treatments. The effect of physical activity has been investigated by Hornig *et al.* [60], who studied the effects of physical training on radial FMD in patients with chronic heart failure. After 4 weeks handgrip training, FMD improved significantly in the trained dominant arm, but not in the nondominant arm, suggesting a local beneficial mechanism.

The most striking reduction in the all-cause mortality in secondary prevention due to reduction in coronary heart disease (up to 70%) has been achieved in a trial that used only dietary modification [61]. Therefore, it is surprising that the potential effects of dietary elements, such as fatty acids, on endothelial function have not been investigated more extensively.

Limitations of the ultrasound method

No longitudinal studies in humans have yet proven that those young subjects with endothelial dysfunction will go on to develop advanced atherosclerosis, since such studies would take decades to complete. Despite this, endothelial dysfunction is spatially and temporally linked to atherosclerosis, occurring first at coronary branch points where plaques tend to develop [62] and preceding occlusive arterial disease [63]. Thus, there are much data to support a link between arterial endothelial dysfunction and later advanced atherosclerotic disease and the use of FMD as a surrogate end-point in clinical trials appears justified. Furthermore, FMD offers an assessment of endothelial function in conduit arteries, which are the vessels prone to the development of atherosclerosis.

Relative disadvantages of the ultrasound technique are that it is difficult to perform, requiring a skilled sonographer and an appropriate training period, and that the 'error' of the method probably precludes serial study of endothelial function for individuals. Therefore, we currently recommend these techniques be used in clinical research, rather than in routine clinical practice. Future developments are likely to enhance the image quality during peripheral arterial scanning, to automate and improve analytical methods for measuring arterial diameter [64] and to link abnormalities observed using this method with cardiovascular event rates.

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